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Inference of Target Gene Regulation via miRNAs during Cell Senescence by Using the MiRaGE Server

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ABSTRACT: miRNAs have recently been shown to play a key role in cell senescence, by downregulating target genes. Thus, inference of those miRNAs that critically downregulate target genes is important. However, inference of target gene regulation by miRNAs is difficult and is often achieved simply by investigating significant upregulation during cell senescence. Here, we inferred the regulation of target genes by miRNAs, using the recently developed MiRaGE server, together with the change in miRNA expression during fibroblast IMR90 cell senescence. We revealed that the simultaneous consideration of 2 criteria, the up(down)regulation and the down(up) regulatiion of target genes, yields more feasible miRNA, i.e., those that are most frequently reported to be down/upregulated and/or to possess biological backgrounds that induce cell senescence. Thus, when analyzing miRNAs that critically contribute to cell senescence, it is important to consider the level of target gene regulation, simultaneously with the change in miRNA expression.

Key words: bioinformatics, mirna, target gene, expression regulation, senescence

MicroRNAs (miRNAs) are small non-coding RNAs, which play important roles in a variety of biological processes. miRNAs negatively regulate the expression of specific target genes at the post-transcriptonal level [1]. A single miRNA has the potential to regulate hundreds of mRNAs, and therefore it is conceivable that miRNAs are important regulatory molecules in complex biological processes such as aging and cancer. miRNAs were recently reported to play a critical role in the control of cell senescence [2]. Neverthless, effective experimental procedures to directly evaluate target gene regulation by miRNAs have not yet been developed. Target genes themselves are often computationally predicted [3], and are therefore usually considered to include a relatively large number of false positives. The restricted accuracy of the target gene prediction means that critical miRNAs for cell senescence are usually selected from among those miRNAs showing high expression in senescent cells, without considering target gene expression [4]. Hackl *et al* [5] reported that some miRNAs, including members of the miRNA-17-92 cluster, were significantly downregulated during cell senescence. This was the first indication of the biological importance of miRNAs not upregulated during cell senescence. More recently, Wang *et al* [6] demonstrated the induction of cell senescence, following the suppression of downregulated miRNAs. Thus, miRNAs may contribute to the progress of cell senescence progression, even when they are not expressed in senescent cells.

Using next generation sequencing (NGS) Dhahbi *et al* [7] identified many more miRNAs up/downregulated during cell senescence than were previously shown to exist by means of microarray analysis. This ability of NGS to detect such a large number of up/downregulated miRNAs generates the following questions. If the number of up/downregulated miRNAs is in the hundreds, which miRNA is the most important? Moreover, is the induction of cell senescence dependent on the up/downregulation of most of these hundreds of miRNAs? Dhahbi *et al* [7] demonstrated that a set of

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genes targeted by up/downregulated miRNAs is biologically informative. However, the importance of individual miRNAs during cell senescence remains to be elucidated.

Here, we propose a procedure for ranking miRNAs based not only on fold changes during cell senescence, but also on criticality during cell senescence: This ranking of miRNAs based upon the level of target gene regulation facilitates the detection of "important" miRNAs among hundreds of up/downregulated miRNAs.

MATERIALS AND METHODS

mRNA expression

mRNA expression profiles during fibroblast cell senescence (accession numbers GEO ID: GSE19018 and GEO ID: GSE15919) were downloaded from the gene expression omnibus (GEO). GEO ID: GSE19018 and GEO ID: GSE15919 represent IMR90 cell lines MRC5 cell lines, respectively. CEL files downloaded from the data set GEO ID: GSE19018 were treated by rma function, using the affy package in Bioconductor R [8]. For young samples, IMR90 cell lines at PD 30 under 20% oxygen (GEO ID: GSM4870491, GEO ID: GSM470492, and GEO ID: GSM470493) were employed. Cell lines at PD 48 (GEO ID: GSM4870494, GEO ID: GSM470495, and GEO ID: GSM470496) were considered to be senescent samples. The raw files were downloaded from data set GEO ID: GSE15919. We used a 2-color array and treated each dye as an independent sample. Thus, MRC5 fibroblasts at PDL 28 (F532 median of GEO ID: GSM399555, GEO ID: GSM399561, and GEO ID: GSM399570 were considered; and F635 median of GEO ID: GSM399560, GEO ID: GSM399569, and GEO ID: GSM399581) were considered to be young samples. Senescent samples comprised those at PDL 63 (F635 median of GEO ID: GSM399555, GEO ID: GSM399561, and GEO ID: GSM399570; and F532 median of GEO ID: GSM399560, GEO ID: GSM399569, and GEO ID: GSM399581).

Transformation of gene expression using principal component analysis and linear discriminant analysis

In order to extract the components that retrieve the critical difference between young and senescent samples, we transformed the gene expression profiles as follows. First, we applied principal component analysis (PCA), and attributed principal component scores to each sample. Next, we employed linear discriminant analysis (LDA) to discriminate between young and senescent samples with an optimal number of principal components (see Tables S1 and S2 in the Supplementary Material (Supp1)). Finally, we obtained a transformed expression x_g corresponding to the gene

$$x_g = \sum_i a_{gi} b_i \quad (1)$$

$$L_s = \sum_i b_i PCS_{is} \quad (2)$$

$$PCS_i = \sum_g a_{gi} x_{gs} \quad (3)$$

where PCS_is is the ith principal component score attributed to the sth sample used for discriminations and x_{gs} is the expression of gene g in the sth sample.

L_s is the discriminant function such that positive or negative L_s values mean senescent or young samples, respectively. We performed all of our analyses using several packages in R.

Inference of target gene expression via the MiRaGE server

The target gene regulation by miRNAs was inferred by the MiRaGE server [11]. Gene expression in young samples was taken to be unit, while that in senescent samples was considered to be $\exp(x_g)$. The values were then uploaded to the MiRaGE server. All options other than "Select the conservation of miRNA", which was taken to be "all", are defaults.

miRNA expression extraction from sequencing data

Fastq files of GEO ID: GSE27404 were downloaded from the ftp server, which can be found at ftp://ftp.sra.ebi.ac.uk/vol1/fastq/SRR107/; the accession numbers were SRA ID: SRR107296 and SRA ID: SRR107297 for young and senescent IMR90 cell lines, respectively. The young (senescent) sample was the IMR90 cell line at PD 14 (34). The fastq files obtained were treated by miRDeep2 [9].

Reads were aligned using mapper.pl to hg19 human genome, with trimming of adapter sequence TCGTATG CCGTCTTCTGCTTGT. The arf and fastq files obtained were analyzed by miRDeep2.pl. The actual command line inputs are detailed in Supplementary Material (Supp1) and the full sets of outputs are available at web links:http://www.granular.com/AD/Supp1.zip (senescent cell results) and http://www.granular.com/AD/Supp2.zip (young cell results). Within them, the csv files for miRNA expression were used for further analysis.

Statistical validation

To determine the validity of inference by the MiRaGE server, we applied the statistical tests detailed in the Supplementary Document.

Table 1. miRNAs downregulated during cell senescence

	Senescent		Young			
miRNA	NMRC	SCORE	NMRC	SCORE	*P*-value	RFC
hsa-miR-143-5p	1.41E-04	7359.9	5.87E-04	24337.1	1.91E-02	4.16
hsa-miR-155-5p	1.30E-04	---	1.32E-03	7329.8	2.29E-02	10.18
hsa-miR-16-5p	3.53E-05	---	3.57E-04	1997.6	1.65E-03	10.10
hsa-miR-199a-3p	3.08E-02	157153.4	8.29E-02	460656.4	1.55E-02	2.69
hsa-miR-199b-3p	3.08E-02	156633.7	8.29E-02	460091.3	1.55E-02	2.69
hsa-miR-214-3p	1.55E-04	---	2.02E-04	1140.1	6.89E-03	1.30
hsa-miR-27a-5p	3.82E-06	5148.3	6.57E-06	2762.3	1.68E-04	1.72
hsa-miR-423-3p	3.50E-04	36845.1	7.10E-04	36425.6	3.33E-03	2.03
hsa-miR-424-5p	1.73E-04	2241.8	6.25E-04	7024.9	1.65E-03	3.62
hsa-miR-503	9.74E-04	4960.8	2.00E-03	11108.8	1.67E-02	2.05

If the rejection probability for target gene upgulation is not significant, i.e., not less than 0.05, the miRNA is omitted. RFC: reciprocal fold change during cell senescence (larger values indicate upregulation in young cells). NMRC: normalized mature read count such that summation over all over miRNAs is taken to be unit. SCORE: miRDeep2 score. *P*-value: the rejection probabilities for target gene upregulation during cell senescence (smaller values indicate more plausible target gene upregulation in senescent cells), computed using the *t* test implemented in the MiRaGE server.

Coincidence of miRNA rankings between the IMR90 and MRC5 cell lines, based upon P-values

We checked for a significant coincidence between the miRNA rankings for the IMR90 cell lines and MRC5 cell lines. *P*-values were computed via binomial distribution

$$P(x, N, \frac{N}{N_{all}})$$

where $P(x, N, \frac{N}{N_{all}})$ is the binomial distribution function where the number of common miRNAs is greater than x, when N represents the number of top-ranked miRNAs considered for each of the cell lines and N_{all} represents the total number of miRNAs considered.

Correlation coefficients between the senescence-associated miRNA expression change and the rejection probability for target gene regulation

The miRNA expression in young cells was subtracted from the miRNA expression in senescent cells. The resulting senescence-associated miRNA expression changes were compared with the rejection probability for target gene regulation, obtained using the MiRaGE server. The miRNA was excluded from the analysis when the miRDeep2 scores in young cells and senescent cells were less than the threshold value. The miRNA was also ignored when the rejection probability for target gene regulation was not significant, i.e., not less than 0.05. Pearson's correlation coefficients and associated *P*-values were computed between the miRNA expression changes and the rejection probabilities for target gene regulation, for a series of threshold values: none, 0, 1, 10, . . . , 10^4 .

RESULTS

First, we attributed a *P*-value to each miRNA via the MiRaGE server [10]. The *P*-values, i.e., the rejection probabilities of target gene regulation, are measures of the likelihood of target gene up/downregulation, if each miRNA does not regulate target genes. Thus, smaller P -values indicate more plausible target gene regulation by each miRNA. Statistical validation revealed that top-ranked miRNAs were often common between IMR90 and MRC5 cell lines ($P<10^{-10}$, see Figure S1-S3 in Supplementary Material (Supp1)). Given that these 2 rankings were obtained from 2 different experiments for the distinct cell lines, this remarkable coincidence indicates the validity of our bioinformatic analysis. To our knowledge, this is the first report of common miRNAs regulating target gene expression in 2 distinct cell lines.

Next we calculated the correlation coefficients between the *P*-values obtained, and the senescence-associated miRNA expression changes for IMR90 cell lines computed according to the NGS data of Dhahbi *et*

al [7]. We revealed that the *P*-values associated with the correlation coefficients were often significant (typically $P<10^{-2}$, see Figure S4-S6 in Supplementary material (Supp1)). Thus, we concluded that the senescence-associated miRNA expression changes match the rejection probabilities of target gene regulation (these data are fully available as supplementary material (Supp2)). To our knowledge, this is the first report of a significant correlation between individual miRNA expression and target gene expression during cell senescence.

Table 2. miRNAs upregulated during the cell senescence

	Senescent		Young			
miRNA	NMRC	SCORE	NMRC	SCORE	*P*-value	FC
hsa-let-7a-5p	1.71E-01	452998.2	1.44E-01	436459.4	3.16E-05	1.19
hsa-let-7c	8.82E-02	---	8.68E-02	12895.9	3.16E-05	1.02
hsa-let-7e-5p	9.18E-02	120470.8	7.33E-02	63861.9	3.16E-05	1.25
hsa-let-7f-5p	1.47E-01	357312.1	1.29E-01	348238.8	3.16E-05	1.14
hsa-let-7i-5p	1.72E-02	87436.7	1.17E-02	65067.1	3.16E-05	1.47
hsa-miR-10a-3p	5.01E-05	18603.4	4.85E-05	20378.1	2.80E-09	1.03
hsa-miR-125a-5p	6.03E-04	3099.8	3.12E-04	1783.5	2.45E-02	1.93
hsa-miR-125b-5p	3.01E-03	15912	1.91E-03	11299.2	2.45E-02	1.58
hsa-miR-136-5p	2.14E-04	1256.3	2.11E-04	1303.2	2.96E-06	1.01
hsa-miR-154-3p	2.55E-04	1456.4	2.28E-04	1407.5	2.02E-02	1.12
hsa-miR-154-5p	3.05E-05	1456.4	2.46E-05	1407.5	4.48E-02	1.24
hsa-miR-181a-5p	5.08E-03	26334.5	4.17E-03	23599.8	7.51E-04	1.22
hsa-miR-181b-5p	5.89E-03	28475.6	3.63E-03	16195.9	7.51E-04	1.62
hsa-miR-181d	4.70E-03	---	3.04E-03	5376.4	7.51E-04	1.55
hsa-miR-193a-5p	5.05E-04	3001.6	3.07E-04	2608.6	4.81E-04	1.65
hsa-miR-221-3p	7.69E-02	392203.2	4.75E-02	265663.6	7.59E-04	1.62
hsa-miR-23a-3p	3.33E-03	16145.7	1.71E-03	9015	4.64E-02	1.95
hsa-miR-23b-3p	3.09E-03	3526.6	1.59E-03	2876.1	4.64E-02	1.95
hsa-miR-30a-3p	2.62E-03	51409.3	1.06E-03	18348.2	6.54E-04	2.47
hsa-miR-30d-3p	7.04E-06	4247.3	4.17E-06	8382	6.54E-04	1.69
hsa-miR-30e-3p	2.04E-03	2536.6	9.28E-04	3231.5	6.54E-04	2.19
hsa-miR-323b-3p	1.98E-04	1011.8	4.93E-05	---	7.06E-04	4.02
hsa-miR-323b-5p	1.31E-06	1011.8	3.70E-07	---	2.31E-02	3.53
hsa-miR-369-3p	2.79E-03	14688.6	9.57E-04	5621.3	1.67E-02	2.92
hsa-miR-369-5p	9.69E-05	14688.6	5.47E-05	5621.3	1.27E-02	1.77
hsa-miR-382-5p	1.33E-03	7295.2	1.03E-03	6479	3.67E-03	1.29
hsa-miR-409-3p	6.01E-04	---	4.86E-04	3724.5	2.25E-02	1.24
hsa-miR-485-3p	5.62E-04	7025	5.04E-04	5279.9	1.88E-02	1.12
hsa-miR-493-5p	2.68E-04	2595.1	8.84E-05	1050.8	1.70E-03	3.04
hsa-miR-494	4.35E-04	2224.7	2.09E-04	1173.5	2.78E-02	2.08
hsa-miR-495	2.68E-03	13646.1	1.30E-03	7246.8	4.65E-04	2.06
hsa-miR-98	3.76E-04	1524.1	1.87E-04	---	3.16E-05	2.01

FC: fold change during cell senescence (larger values indicate upregulation in senescent cells). *P*-value: the rejection probabilities for target gene downregulation during cell senescence (smaller values indicate more plausible target gene downregulation in senescent cells), computed using the *t* test implemented in the MiRaGE server. Other notations are the same as in Table 1.

DISCUSSION

We have successfully demonstrated that inference of target gene regulation is coincident with miRNA expression changes during cell senescence. Here, we will confirm the validity of our inference when selecting critical miRNAs during cell senescence. Table 1 and 2 list those miRNAs that are down(up)regulated during cell senescence, together with their corresponding target genes that are up(down)regulated. Thus, if the miRNA is down(up)regulated during cell senescence, the rejection probability of the corresponding target genes being up(down)regulated should be small. The threshold miRDeep2 score is taken to be 10^3, such that the *P*-value associated with the correlation coefficient is at a minimum. Table 1 lists those miRNAs that are known to be downregulated during cell senescence. miR-155-5p is downregulated in renal proximal tubule epithelial cells (RPTECs), T-cells (organismal aging [OA]), and human dermal fibroblasts (HDFs). miR-16 is downregulated in HDFs, human umbilical vein endothelial cells (HUVECs), RPTECs, and mesenchymal stem cells (MSCs). miR-199a/b-3p and miR-214 are downregulated in foreskin cells. miR-27a is downregulated in T-cells (OA, replicative aging [RA]). miR-423-3p is downregulated in foreskin cells and T-cells (RA). miR-424 is downregulated in MSC, foreskin cells, and RPTECs. All in this paragraph is listed in Table S2 in Hackl *et al's* paper [5].

Cell senescence-associated downregultion of miR-143 and miR-503 has not previously been reported. However, Khach Lai *et al* [11] demonstrated that miR-143 downregulation induced cell-cycle arrest in MSCs. Given that cell-cycle arrest is the cause of cell senescence, this finding is indicative of miR-143 downregulation matches the miR-143 downregulation during cell senescence. miR-503 downregulation induced apoptosis in MSC [12]. Apoptosis and cell senescence are often related, and may even be caused by the same protein, e.g., TGF-β [13,14]. Thus, it appears that miR-503 is also downregulated during cell senescence. miR-155 was previously reported to be downregulated in senescent human fibroblasts [15] and senescent human foreskin fibroblasts cell lines called BJ [16] while miR-27b and miR-199a were shown to be downregulated in the MSCs from elderly patients [17]. Table 2 lists those miRNAs that are known to be upregulated during cell senescence. Let-7c and Let-7f are upregulated in senescent fibroids [18]. miR-136, miR409-3p, and miR495 are upregulated in senescent WI-38 cells [6,19]. miR-30 is upregulated during induced and replicative senescence [20], while miR-23a and miR-23b are upregulated in T-cells (OA/RA) in Table S2 in Hackl *et al's* paper [5]. miR-221 has not previously been reported to be upregulated during cell senescence. However, Chen *et al* [21] demonstrated that miR-221 upregulates GAX, which is known to cause cell senescence. Overexpression of miR-125 and miR-181 was shown to induce cell senescence via downregulation of Cbx7 [22], while mir-125b is known to induce senescence in human melanoma cells [23]. Our findings regarding most of the miRNAs listed in Tables 1 and 2 have experimentally been validated. Moreover, it is likely that supporting evidence will soon be available for the remaining miRNAs, because new findings are reported on a frequent basis.

Finally, it is important to recognize that not all miRNAs undergo more than 2 fold changes. This suggests that, not only fold changes, but also target gene regulations, should be considered when further investigating the correlation between miRNA expression and target gene expression during cell senescence.

In Conclusion, we propose that inference of target gene regulation by miRNAs, together with the miRNA expression change during cell senescence, should be used to estimate critical miRNAs during cell senescence. Liang *et al* [24] reported that miRNA expression does not always reflect miRNA activities. Thus, when selecting critical miRNAs, it is insufficient to only consider the miRNA expression change. Our strategy of screening candidate miRNAs by simultaneously considering target gene regulation and miRNA expression of particular value when using NGS to measure miRNA expression, In comparison with microarray analysis, NGS facilities the detection of more subtle changes during cell senescence, and yields more candidates for critical miRNAs.

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Supplementary Document

Supplementary Material (Supp1)
www.aginganddisease.org/supp1.pdf
Supplementary Material (Supp2)
(www.aginganddisease.org/Supp2.pdf)

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